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Original contribution

Decreased roundabout 1 expression promotes development of intrahepatic cholangiocarcinoma☆

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Summary Roundabout 1 (Robo1) is a transmembrane receptor of the immunoglobulin family. Slit2 is one of its ligands. The function of Slit2/Robo1 signaling in the development of intrahepatic cholangiocarcinoma (ICC) remains to be elucidated. We examined the immunohistochemical expression of Robo1 and Slit2 and their clinicopathologic implications in 132 cases of ICC. Also, small interfering RNA of Robo1 was transfected into a high-expression ICC cell line, and a Robo1 vector was transfected into a low–Robo1 expression ICC cell line. The effect of Robo1 suppression and overexpression in cell proliferation and migration of cultured ICC cells with Slit2 stimulation was investigated. Immunohistochemical study of ICC in the low–Robo1 expression group showed larger tumors (P = .015), a higher Ki-67 labeling index (P = .021), and low expression of Slit2 (P = .0005). The low–Slit2 expression group frequently showed perineural invasion (P = .036) and lymph node metastases (P = .013). Low Robo1 expression was associated with a poor prognosis (P = .0207). Robo1 suppression in Huh28 cells tended to promote cell proliferation and migration, whereas Robo1 overexpression in RBE cells significantly suppressed cell proliferation and migration. Low Robo1 expression was associated with cell proliferation and migration in ICC and was one of the adverse prognostic factors in patients with these tumors.

1. Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer. It is rare, accounting for only 5% to 10% of primary liver cancers [1]. At the time of diagnosis, patients frequently have disease beyond the limits of surgical therapy in the form of lymph node (LN) metastases, peritoneal dissemination, or distant metastases [1,2]. New therapeutic targets are required to improve the survival rates of patients with ICC.

Abbreviations: DFS, disease-free survival; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICC, intrahepatic cholangiocarcinoma; LI, labeling index; OS, overall survival; Robo1, roundabout 1; RT-PCR, reverse transcriptase polymerase chain reaction.

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Roundabout 1 (Robo1) is a transmembrane receptor of the immunoglobulin family consisting of an extracellular domain containing 5 immunoglobulin regions and 3 fibrinogen type III repeats, and an intracellular domain containing CC0, CC1, CC2, and CC3 motifs [3,4]. Slit2 is one of the ligands of Robo1 and interacts with it to mediate repulsive cues in axon guidance and neuronal migration. Slit2/Robo1 signaling in cancer cells suppresses the progression and migration of breast [5], lung [5,6], colorectal [7], and cervical cancers [8] and glioma [9]. Slit2/Robo1 signaling can also promote progression in hepatocellular carcinoma [10] and gastric cancer [11]. However, no studies have yet been conducted on the role of Slit2 and Robo1 in ICC.

In this study, we examined by immunohistochemical analysis the correlation between Slit2 and Robo1 expression and clinicopathologic findings in resected ICCs. We also investigated the effect of Robo1 on proliferation and migration of cultured ICC cells.

2. Materials and methods

2.1. Patients and samples

Paraffin-embedded specimens from 132 patients with ICC who underwent hepatectomy between 1984 and December 2004 at our institute were retrieved. Any patients undergoing previous therapy or noncurative surgery and any patients with extrahepatic bile duct tumors were excluded. Samples from 85 men and 47 women with a mean age of 64 years (range, 33-90 years; median, 65 years) were found. The median follow-up was 991 days (range, 7-5475 days).

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of Kyushu University Hospital (No. 21-118). Informed consent was obtained from each patient in the study.

2.2. Immunohistochemistry

Sections of resected specimens were fixed in 10% buffered formalin, embedded in paraffin, and stained using an EnVision+ System and DAB Kit (DAKO, Glostrup, Denmark). Immunohistochemical stains were performed with antibodies to Robo1 (rabbit polyclonal 1:1000; Life-Span BioSciences, Seattle, WA), Slit2 (goat polyclonal 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), or Ki-67 (MIB-1 1:200; DAKO). Before being incubated with the primary antibodies, sections were treated in a microwave oven at 99°C for 20 minutes for Slit2 or in a pressure cooker for 25 minutes for Ki-67. Robo1 was stained without any heat treatment.

Each slide was stained in serial sections and examined by 2 pathologists (Y.M. and S.A.). In cytoplasmic staining of Robo1 and Slit2 and nuclear staining of Ki-67, the percentages of positive cells were estimated by a count of 1000 tumor cells in the most staining areas (hot spots). A consensus judgment was adopted for the immunohistochemical score of the tumors based on the strength of Robo1 and Slit2 expression: 0, negative; 1+, weak staining; 2+, moderate staining; or 3+, strong staining. The distribution of positive cells was also recorded to portray the diffuse or focal nature of the staining: sporadic (positive cells <10%), focal (positive cells ≥11% but <50%), or diffuse (positive cells ≥50%). Samples with immunohistochemical scores of 2+ and 3+ with focal to diffuse distributions were considered to represent high expression of Robo1 and Slit2. We also examined Robo1 expression in the LN metastases in 19 cases.

2.3. Cell lines

Human ICC cell lines RBE, ssp25, Huh28, and TKKK were obtained from RIKEN BioResource Center, Ibaraki, Japan, and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. All in vitro experiments were performed in triplicate.

2.4. Immunoblotting

Cellular proteins were solubilized in lysis buffer containing protease inhibitor. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane. After blocking in Tris buffer containing 2% bovine serum albumin, the membrane was stained with a 1:2000 dilution of anti-Robo1 and anti-Slit2 antibodies, then washed and incubated with horseradish peroxidase–conjugated secondary antibody (Cell Signaling Technology, Boston, MA). Bands were visualized and enhanced using a chemiluminescence system (GE Healthcare, Buckinghamshire, UK).

2.5. Real-time reverse transcriptase polymerase chain reaction

Semiquantitative reverse transcriptase polymerase chain reaction (PCR) for Robo1 messenger RNA (mRNA) was performed on 18 frozen samples of ICC using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Total RNA of the frozen samples of ICC, HeLa cells, and MCF cells was extracted using Trizol reagent (GIBCO BRL, Rockville, MD), and reverse transcription was performed with QuantiTect Reverse Transcription (Qiagen, Hilden, Germany). A *Taq*Man probe for Robo1 (Hs00268049_m1) and a set of primers and a probe for the internal housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; *Taq*Man GAPDH control reagent kit) were purchased from Perkin-Elmer Applied Biosystems (Waltham, MA). The PCR was performed in a 50-μL mixture containing 25 μL of 2× *Taq*Man Universal PCR Master Mix